**Report Title: Test of the Potential of Octopirox to Induce Mutation in the Skin of Muta™ Mouse *In Vivo.***

**Test Type:** Genotoxicity Study

**Conducting Laboratory and Location:** P&G Miami Valley Laboratories, Biological Testing Facility, Cincinnati, OH

**Test Substance(s):** G0539.06 – Octopirox in ethanol

**Species:** Muta Mouse *In Vivo*

**# of Animals:** 3 mice per group

**Test Conditions:** The potential of Octopirox to induce mutations in skin was evaluated using Muta Mouse. This transgenic strain was developed to allow the detection of mutation in any tissue *in vivo*. A single application of 0.1ml of a 7.5% solution of OP in ethanol was topically applied to Muta Mouse skin. This dose was previously determined to be a maximum tolerated dose.

**Results:** The potential of Octopirox to induce mutations in skin was evaluated using Muta Mouse. This transgenic strain was developed to allow the detection of mutation in any tissue *in vivo*. The maximum tolerated dose was not mutagenic though in previous studies it had been shown to inhibit DNA synthesis in the Muta Mouse. See study #B91-0153.

**Study #:** B91-0227
**Report Date:** 4/27/92
**QA report/GLP compliance**: Yes

# Test of the Potential of Octopirox to Induce Mutation in the Skin of Muta™Mouse *In vivo*
**B91-0227**

Robert L. Binder, Audrey A. Erickson, Roman E. Frank and E. D. Thompson

Human and Environmental Safety Division
Miami Valley Laboratories
The Procter & Gamble Company
Cincinnati, OH 45239

Report Date 4/27/92

Procter & Gamble

The Procter & Gamble Company
Miami Valley Laboratories
P. O. Box 398707 Cincinnati, Ohio 45239-8707

# QUALITY ASSURANCE STATEMENT

| | |
|---|---|
| STUDY NUMBER | B91-0227 |
| TEST FACILITY | The Procter & Gamble Company<br>Miami Valley Laboratories<br>Cincinnati, Ohio 45239 |
| TYPE OF STUDY | Test of the Potential of Octopirox to Induce Mutation in the Skin of MutaMouse In Vivo |
| DIVISIONAL REQUEST DOCUMENT | None |
| TSIN | G0539.06, R0363.01, R0362.01 |
| DATA LOCATION | YB-1402 |

| PORTION(S) OF STUDY AUDITED: | AUDITOR | DATE AUDITED: | DATE REPORTED TO STUDY DIRECTOR: | DATE REPORTED TO MAN-AGEMEMT: |
|---|---|---|---|---|
| Dosing | L. K. Klahn | 6/28/91 | 7/18/91 | 8/27/91 |
| 4-Day Sacrifice | L. K. Klahn | 7/2/91 | 7/18/91 | 8/27/91 |
| Study Data | L. K. Klahn | 7/18/91 | 7/18/91 | 8/27/91 |

In compliance with the Good Laboratory Practices regulations this study has been audited by the Quality Assurance Unit and the results of those audits have been reported to the appropriate management. The protocol was audited for GLP required elements. The study data accurately reflects the procedures described in the protocol. The reported results accurately reflect the raw data of the study.

Ryan K. Klahn 4/29/92
Quality Assurance Unit - Date

## SUMMARY SHEET

| | |
|---|---|
| Study No. | B91-0227 |
| Animal Activity No. | AA91-0081 |
| Testing Facility: | Biological Testing Facility<br>Miami Valley Laboratories<br>The Procter and Gamble Co.<br>P.O. Box 398707<br>Cincinnati, OH 45239 |
| Test Substance(s): | Octopirox (TSIN G0539.06)<br>Hydroxyurea (TSIN R0363.01)<br>7,12-Dimethylbenzanthracene (TSIN R0362.01) |
| Storage Conditions: | Octopirox (room temp.), hydroxyurea (refrigerated), 7,12-dimethylbenz-anthracene (approx.-20°C in dark) |
| DRD: | HESE 360 |
| Date Study Started: | 6/26/91 (Mice first shaved) |
| Date Study completed (in-life): | 7/12/91 |
| Report Date: | 4/27/92 |
| Study Director: | Robert L. Binder |
| Study Technicians: | Audrey A. Erickson<br>Roman E. Frank |
| Genetic Toxicologist: | Edward D. Thompson |
| Notebook: | YB-1402 |
| Archived at: | Miami Valley Laboratories |

CONTENTS

## I. PURPOSE

The purpose of this study was to determine whether a single maximum tolerated dose of Octopirox, applied topically, is mutagenic in mouse skin.

## II. SUMMARY

The potential of Octopirox to induce mutations in skin was evaluated using Muta™Mouse. This transgenic strain was developed to allow the detection of mutation in any tissue *in vivo* (1,2). Octopirox was topically applied to Muta™Mouse skin in ethanol at a dose of 7.5 mg (~1100 $\mu g/cm^2$), which previously was determined to be a maximum tolerated dose (3). The potential of hydroxyurea (HU) to induce mutations in skin was also evaluated for comparison to Octopirox, and the established skin mutagen, 7,12-dimethylbenzanthracene (DMBA), was used as a positive control.

Since the optimal time after dosing to sample skin for mutation analysis was not known, skin samples from mice killed 4, 7 and 10 days after dosing with 100 $\mu g$ of DMBA were analyzed for mutations. At 4 days after dosing with DMBA the mean mutant frequency was about twice that in controls. In contrast, at 7 and 10 days after dosing the mutant frequencies were about 13-times the control level. Therefore, both 7 and 10 days after dosing were appropriate times to collect skin for mutation analysis. At these times the mutant frequencies in skin treated with Octopirox were not greater than those in solvent controls. Also, 7 days after dosing, hydroxyurea (0.5 mg/g body weight i.p.) did not induce an increase in mutant frequency. Therefore, under the conditions of testing both Octopirox and hydroxyurea were not mutagenic *in vivo*.

Both Octopirox and hydroxyurea had been shown previously to cause a > 90% inhibition of epidermal DNA synthesis in Muta™Mouse skin at the doses shown here not to be mutagenic. Therefore, the transient inhibition of epidermal DNA synthesis by a single high dose of either Octopirox or hydroxyurea is not associated with mutagenesis in Muta™Mouse.

## III. METHODS

### Materials

Octopirox (TSIN G0539.06) was obtained from Beauty Care Product Development. Hydroxyurea (TSIN R0363.01) was obtained from the Sigma Chemical Co., and DMBA (TSIN R0362.01) was from the Aldrich Chemical Co. Other chemicals were of reagent grade or higher quality and their sources are indicated in the study notebook.

### Animals

Male mice of the Muta™Mouse strain were received from Hazleton Research Laboratories at approximately 7 weeks of age, and were housed 5/shoebox cage on hardwood chip bedding. A 12 hr light/dark cycle (7:00 am to 7:00 pm) was maintained in the animal rooms (with the exception noted below), and Purina Lab Chow and water were available *ad libitum*. Room temperature and humidity were maintained to Biological Testing Facility (BTF) standards (BTF SOP: ENV 3.4). The mice were carefully shaved when they were 8

to 8 weeks old using a small animal clipper, and only mice in the resting phase of the hair cycle (i.e. animals without obvious hair regrowth within two days of shaving) were used. Mice were individually housed after shaving and treatments did not begin until at least 2 days after shaving. Initially all mice were maintained in room L-42 of the BTF. However, mice to be treated with DMBA or HU were transferred to room L-43 in the Carcinogen Area after shaving, and remained there until the end of the experiment.

**Dosing**

The overall experimental schedule and identity of groups are indicated below. Mice were uniquely identified with the group numbers (1-4) and letters (A-C) and animal numbers (1-3), which were written on their tails with a permanent marker before dosing. The Muta™Mouse strain has 3 coat colors: black, brown and golden brown. Mice of different colors were distributed as uniformly as possible among the various treatment groups.

| Group | Animals | Treatment | Days Between Treatment and Killing |
|---|---|---|---|
| 1A | 1-3 | 0.1 ml ethanol (EtOH | 4 |
| 1B | 1-3 | 0.1 ml EtOH | 7 |
| 1C | 1-3 | 0.1 ml EtOH | 10 |
| 2A | 1-3 | 7.5 mg Octopirox (Octo | 4 |
| 2B | 1-3 | 7.5 mg Octo | 7 |
| 2C | 1-3 | 7.5 mg Octo | 10 |
| 3A | 1-3 | 0.5 mg/g Hydroxyurea (HU) | 4 |
| 3B | 1-3 | 0.5 mg/g HU | 7 |
| 3C | 1-3 | 0.5 mg/g HU | 10 |
| 4A | 1-3 | 100 μg DMBA | 4 |
| 4B | 1-3 | 100 μg DMBA | 7 |
| 4C | 1-3 | 100 μg DMBA | 10 |

Group 1 was treated with 0.1 ml of ethanol and group 2 received a single application of 0.1 ml of a 75 mg/ml (7.5%) solution of Octopirox in ethanol. The ethanol or Octopirox solution was dripped over the shaved area using a micropipettor to achieve uniform coverage, while avoiding the border so that the dose was not wicked into the surrounding hair.

Mice in group 3 received 0.5 mg hydroxyurea/g body weight dissolved in isotonic saline (150 mg hydroxyurea/ml) by sterile i.p. injection.

Mice in group 4 received a single topical application of 100 μg of DMBA in 0.1 ml of acetone (1 mg/ml). The dose was dripped on the skin to achieve uniform coverage as indicated above for ethanol or the Octopirox solution. During dosing with DMBA and for approximately the next 1.5 hr the mice were maintained under yellow lights, then the white room lights were turned back on. Because of an error in setting the light switches in the room (L-43), the timer was bypassed and the lights remained on overnight. The

problem was corrected the following day. Groups 1 and 2 were housed in a separate room from groups 3 and 4 and were not affected.

Since the altered light cycle potentially could affect the circadian rhythm of epidermal DNA synthesis, additional mice were dosed with DMBA and HU as indicated below. Again dosing with DMBA was done under yellow light.

| Group | Animals | Treatment | Days Between Treatment and Killing |
|---|---|---|---|
| 3D | 1-3 | 0.5 mg/g HU | 7 |
| 4D | 1,2 | 100 μg DMBA | 7 |

All necessary precautions were taken to avoid any possible cross contamination of mice in the different treatment groups. Groups 3 and 4, while maintained in the same room, were kept physically separated on different cage racks in independent cage ventilation chambers.

**Tissue Collection**

At the times after dosing indicated above, mice were killed by $CO_2$ asphyxiation. The treated skin was excised, leaving a border to ensure only treated skin was sampled. Dissection instruments were carefully washed after each mouse to ensure there was no cross contamination of samples. Also, the mice were killed in the order of groups 1 -> 4 which minimized the significance of any inadvertent cross contamination. The skin samples were sealed in labelled plastic bags, frozen in liquid nitrogen, and stored at -80° C until sent to Hazleton Research Laboratories for mutation analysis.

**Safety Considerations**

Handling of all test materials was consistent with personnel protection and the SOP for the Carcinogen Laboratory (Standard Method MCM-26). All cages, cage tops and water bottles from mice treated with DMBA were disposed of as carcinogenic waste. DMBA-treated mice were dissected in the chemical fume hood in L-43 (BTF). Dissection instruments and carcasses were discarded as carcinogenic waste.

**Analysis of Mutations in Skin**

Analysis of mutations in the skin samples was done at Hazleton Laboratories. The samples analyzed were: 1B(1-3), 1C(1-3), 2B(1-3), 2C(1-3), 3D(1-3), 4A(1-3), 4B2, 4C(1-3), 4D(1,2). Samples 1A (1-3) and 2A (1-3), collected 4 days after dosing, were not analyzed because the positive control, DMBA, induced a much lower mutant frequency at 4 days after dosing compared to the effects at 7 and 10 days after dosing.

The methods for analysis of mutations in the skin samples are described in detail in Appendix I, the report from Hazleton Laboratories.

## V. RESULTS

Results of mutation assays are described in detail in Appendix I and summarized below.

### DMBA

Some crusting and focal ulceration of treated skin was noted 7 days after dosing mice with 100 $\mu$g DMBA.

The skin mutation assay results from the DMBA-treated mice are shown in Table 1. Approximately 300,000 plaques were scored, since previous experience indicated that this was adequate to detect the strong response induced by this potent mutagen and carcinogen. DMBA was dosed in acetone, and a specific vehicle control for DMBA was not included in the experimental design. However the mutant frequencies in skin treated with ethanol, the vehicle for Octopirox, are shown in Table 2. These values ranged from 23.8 X $10^{-6}$ to 55.9 X $10^{-6}$ with an overall mean value obtained at 7 and 10 days after dosing of 40.1 X $10^{-6}$. Similar mutant frequencies have been reported in the skin of untreated and acetone treated mice (2).

Since the optimal time after dosing to sample skin for mutation analysis was not known, skin samples from mice killed 4, 7 and 10 days after dosing with DMBA were analyzed for mutations. At 4 days after dosing the mutant frequency in DMBA-treated mice was about twice that in ethanol controls. However, at 7 and 10 days the mutant frequencies were about 13-times the mean level in ethanol controls, indicating that both 7 and 10 days after dosing were appropriate times to collect skin for mutation analysis.

Mouse 4D1, sampled at 7 days after dosing, had a mutant frequency of about 7000 X $10^{-6}$, which is more than 10-fold greater than that determined in the skin of the other DMBA-treated mice. As discussed in Appendix I, such a high mutant frequency is probably the result of a heritable mutation in the target *lac Z* gene.

As noted in the Methods section most of the DMBA-treated mice were subject to an altered light cycle during the day of dosing. Mouse 4D2 was an additional mouse subsequently dosed and maintained under the normal light cycle and killed 7 days after dosing. The mutant frequency observed in 4D2 was similar to that in 4B2, which was also killed 7 days after dosing. Furthermore, the mutant frequencies determined in all the mice killed 10 days after dosing were similar to the value obtained with 4D2. Therefore, it is unlikely that the alteration in the light cycle affected the induction of mutations in skin by DMBA.

The strong mutation response induced by the positive control, DMBA, indicates that the Muta™Mouse skin mutation assay as performed in this study was capable of detecting a known mutagen and skin carcinogen.

Octopirox and Hydroxyurea

Results from Muta™Mouse skin mutation assays on Octopirox and hydroxyurea are shown in Table 2. Based on the relatively low response to DMBA 4 days after dosing, only skin samples from the mice killed 7 and 10 days after dosing were evaluated for mutation. Approximately, 600,000 plaques were scored, since preliminary validation studies indicated this was an adequate number to assess mutant frequency. At both 7 and 10 days after dosing, the mutant frequencies in skin from mice treated topically with Octopirox were not greater than those in solvent controls. Also, 7 days after dosing hydroxyurea did not induce an increase in mutant frequency. Therefore, under the conditions of testing both Octopirox and hydroxyurea were not mutagenic *in vivo*.

## IV. DISCUSSION

The dose of Octopirox evaluated here for mutagenic potential (7.5 mg or ~1100 $\mu g/cm^2$) (4) was shown previously to be a maximum tolerated dose based on the severity of dermal irritation induced by higher doses (3). This dose was not mutagenic, but in an earlier study caused > 90% inhibition of epidermal DNA synthesis in Muta™Mouse (4). Similarly, the dose of hydroxyurea tested (0.5 mg/g body weight i.p.), caused a 98% inhibition of epidermal DNA synthesis in Muta™Mouse (5), yet was not mutagenic *in vivo*. Together these results indicate that the transient inhibition of epidermal DNA synthesis by a single high dose of either Octopirox or hydroxyurea is not associated with mutagenesis *in vivo*.

Robert L. Binder

4/27/92
Date

Audrey A. Erickson

Roman E. Frank

Edward D. Thompson

## VI. REFERENCES


1. Gossen, J.A., deLeeuw, W.J.F., Tan, C.H.T., Zwarthoff, E.C., Berends, F., Lohman, P.H.M., Knook, D. L., and Vijg, J. (1989) Efficient rescue of integrated shuttle vectors from transgenic mice: A model for studying mutations *in vivo*. Proc. Natl. Acad. Sci. USA **86**: 7971-7975

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3. Binder, R.L., Erickson, A.A., Reer, P.J. and Frank, R.E. (1992) Evaluation of skin irritation potential of acetic acid and Octopirox in the Muta™Mouse. The Procter & Gamble Company, Study B91-0116 Report.

4. Binder, R.L., Erickson, A.A. and Frank, R.E. (1992) Inhibition of epidermal DNA synthesis in Muta™Mouse by Octopirox: time-course and dose-response. The Procter & Gamble Company, Study B91-0153 Report.

5. Binder, R.L., Erickson, A.A. and Frank, R.E. (1992) Inhibition of epidermal DNA synthesis in Muta™Mouse by hydroxyurea. The Procter & Gamble Company, Study B91-0210 Notebook reference YE-1402, pg 85.

TABLE

Muta™Mouse Skin Mutation Assay Results from Mice Treated with 100 μg DMBA

| Anima | Total Plaques | Mutants | M.F. X $10^{-6}$* |
|---|---|---|---|
| | | *4 Days After Dosing* | |
| 4A1 | 316,065 | 23 | 73 |
| 4A2 | 288,384 | 37 | 128 |
| 4A3 | 292,683 | 14 | 48 |
| | | AVG ± SD | 83 ± 41 |
| | | *7 Days After Dosing* | |
| 4B2 | 312,690 | 137 | 438 |
| 4D1 | 139,725 | 97 | Spontaneous Mutant |
| 4D2 | 312,908 | 179 | 572 |
| | | AVG ± SD | 505 ± 95 (N = 2) |
| | | *10 Days After Dosing* | |
| 4C1 | 286,152 | 148 | 517 |
| 4C2 | 300,426 | 193 | 642 |
| 4C3 | 307,510 | 141 | 459 |
| | | AVG ± SD | 539 ± 94 |

*M.F. indicates mutant frequency.

TABLE 2.

Muta™Mouse Skin Mutation Assay Results from Mice Treated with Octopirox and Hydroxyurea

| Animal | Total Plaques | Mutants | | M.F. X $10^{-6}$* |
|---|---|---|---|---|
| | | ***7 Days After Dosing*** | | |
| *Ethanol Control* | | | | |
| 1B1 | 588,015 | 14 | | 23.8 |
| 1B2 | 619,445 | 20 | | 32.3 |
| 1B3 | 591,809 | 22 | | 37.2 |
| | | | AVG ± SD | 31.1 ± 6.8 |
| *Octopirox (7.5 mg)* | | | | |
| 2B1 | 602,998 | 10 | | 16.6 |
| 2B2 | 668,547 | 26 | | 38.9 |
| 2B3 | 672,949 | 23 | | 34.2 |
| | | | AVG ± SD | 29.9 ± 11.8 |
| *Hydroxyurea (0.5 mg/g body wt)* | | | | |
| 3D1 | 627,636 | 14 | | 22.3 |
| 3D2 | 628,494 | 15 | | 23.9 |
| 3D3 | 574,604 | 24 | | 41.8 |
| | | | AVG ± SD | 29.3 ± 10.8 |
| | | ***10 Days After Dosing*** | | |
| *Ethanol Control* | | | | |
| 1C1 | 590,667 | 33 | | 55.9 |
| 1C2 | 611,948 | 28 | | 45.8 |
| 1C3 | 611,523 | 28 | | 45.8 |
| | | | AVG ± SD | 49.2 ± 5.8 |
| *Octopirox (7.5 mg)* | | | | |
| 2C1 | 600,587 | 17 | | 28.3 |
| 2C2 | 707,802 | 21 | | 29.7 |
| 2C3 | 615,523 | 26 | | 42.2 |
| | | | AVG ± SD | 33.4 ± 7.7 |

*M.F. indicates mutant frequency.

APPENDIX

HAZLETON WASHINGTON REPORT

ANALYSIS OF MUTAMOUSE TISSUES FOR LACZ MUTANT FREQUENCY

SKIN SAMPLES FOR STUDY B91-0227

HAZLETON
WASHINGTON
9200 LEESBURG PIKE
VIENNA VIRGINIA 22182

# ANALYSIS OF MUTAMOUSE TISSUES FOR LACZ MUTANT FREQUENCY

## SKIN SAMPLES FOR STUDY B91-022?

## FINAL REPORT

AUTHOR

BRIAN C. MYHR, Ph.D.

PERFORMING LABORATORY

HAZLETON WASHINGTON, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20895
and
9200 LEESBURG PIKE
VIENNA, VIRGINIA 22182

LABORATORY PROJECT ID

HWA STUDY NOS.: 14654-0-300
14654-1-300
14654-2-300

P&G REFERENCE NO.: 91000672

SUBMITTED TO

THE PROCTER & GAMBLE COMPANY
MIAMI VALLEY LABORATORIES
P.O. BOX 398707
CINCINNATI, OHIO 45239-8707

STUDY COMPLETION DATE

March 27, 1992

HAZLETON
WASHINGTON

# QUALITY ASSURANCE STATEMENT

PROJECT TITLE: ANALYSIS OF MUTAMOUSE TISSUES FOR LACZ MUTANT FREQUENCY

PROJECT NO.: 25501 ASSAY NO.: 14654

PROTOCOL NO.: 300 EDITION NO.: 1

Quality Assurance inspections of the study and/or review of the final report of the above referenced project were conducted according to the Standard Operating Procedures of the Quality Assurance Unit and according to the general requirements of the appropriate Good Laboratory Practice regulations. Findings from the inspections and final report review were reported to management and to the study director on the following dates:

| Inspection/Date | Findings Reported | Auditor |
|---|---|---|
| Plaque Scoring/ 8-14-91 | 8-14-91 | W. Yee |
| Final Report Review/ 3-26-92 | 3-27-92 | D. Wallace |

Quality Assurance Unit — 3-27-92 Date Released

14654-0-300
14654-1-300
14654-2-300

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WASHINGTON

## COMPLIANCE AND CERTIFICATION STATEMENT

The described study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Code of Federal Regulations (21 CFR 58, 40 CFR 792, and 40 CFR 160). To the best of the signers' knowledge, there were no significant deviations from the aforementioned regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results. The raw data have been reviewed by the Study Director, who certifies that the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria.

All test and control results presented in this report and the supporting raw data are maintained in the archive files of Hazleton Washington, Inc., Vienna, Virginia. Copies of the raw data will be supplied to the sponsor upon request.

SUBMITTED BY:

LABORATORY SUPERVISOR:

Hoda Khouri, M.S.
Research Associate
Department of Genetic and
Cellular Toxicology

3/27/92
Date

STUDY DIRECTOR:

Brian C. Myhr, Ph.D.
Associate Director
Department of Genetic and
Cellular Toxicology

3/27/92
Study Completion
Date

14654-0-300
14654-1-300
14654-2-300

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## SUMMARY

A CD2 transgenic mouse strain, designated MutaMouse, which contains multiple copies of a lambda gt10lacZ construct integrated into the genome of each cell, was used to evaluate the mutagenic activities of two test articles, G0539.06 and R0363.01. Twenty-four (24) coded samples of skin tissue were provided to Hazleton Washington, Inc., to determine the frequency of lacZ mutants. DNA was extracted from each skin sample, and the lacZ genes were packaged in vitro into lambda pre-heads. The resultant phage were plated onto E. coli C ($lacZ^-$) agarose cultures in the presence of a chromogenic substrate (Xgal) for the lacZ gene product, β-galactosidase. The plaques that subsequently developed were colored blue for the nonmutant, $lacZ^+$ genes, whereas mutations in the lacZ gene were indicated by colorless or light blue plaques. All plaques suspected of being mutants were replated to confirm the mutant phenotype. The ratio of confirmed mutant plaques to the $lacZ^+$ plaques was calculated as the lacZ mutant frequency in each skin sample.

After analysis of the skin samples for the lacZ mutant frequency, the samples were decoded. Six negative control samples, harvested in triplicate at 7 days and 10 days after study initiation, showed mutant frequencies in the range of $23.8 \times 10^{-6}$ to $55.9 \times 10^{-6}$, which were consistent with the Hazleton historical data base for skin. Other samples were exposed to 7,12-dimethylbenz(a)anthracene (DMBA), as a positive control, and harvested in triplicate at 4, 7 and 10 days after treatment. The samples obtained at 7 and 10 days showed large increases in mutant frequency ($505 \times 10^{-6}$ and $539 \times 10^{-6}$, respectively, as averages). Test article G0539.06 showed no increases in mutant frequency for triplicate samples harvested at 7 and 10 days; the individual samples ranged from $16.6 \times 10^{-6}$ to $42.2 \times 10^{-6}$. Likewise, test article R0363.01 showed no increase in mutant frequency at a single harvest time of 7 days. These results demonstrate the lack of mutagenic activity in skin by G0539.06 and R036.01 under test conditions that led to large increases in mutant frequency after treatment with DMBA.

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# ANALYSIS OF MUTAMOUSE TISSUES FOR LACZ MUTANT FREQUENCY

I. STUDY IDENTIFICATION

A. Sponsor: The Procter & Gamble Company

B. Test Articles:

1. Sponsor Test Substance Identification Numbers (TSIN):

G0539.06 and R0363.01

supplied within the following coded skin samples (24):

1B1, 1B2, 1B3, 1C1, 1C2, 1C3, 2B1, 2B2, 2B3, 2C1, 2C2, 2C3, 3D1, 3D2, 3D3, 4A1, 4A2, 4A3, 4B2, 4C1, 4C2, 4C3, 4D1, 4D2

Coded skin samples (6) provided but not analyzed in this report: 1A1, 1A2, 1A3, 2A1, 2A2, 2A3.

2. Sponsor Study Number: B91-0227

3. Physical Description of the Test Samples: Frozen skin samples individually sealed in labeled bags.

4. Dates Received: July 10, July 16, and October 10, 1991

5. Genetics Assay Number: 14654

C. Type of Assay: Analysis of MutaMouse Tissues for LacZ Mutant Frequency

D. Protocol Number: 300, Edition 1

E. Study Dates:

1. Study Initiation Date: July 11, 1991

2. Experimental Start Date: July 23, 1991

3. Experimental Termination Date: December 12, 1991

F. Supervisory Personnel:

1. Laboratory Supervisor: Hoda Khouri, M.S.

2. Study Director: Brian Myhr, Ph.D.

## II. OBJECTIVE

The objective of this study was to evaluate the lacZ mutant frequency in MutaMouse skin samples provided under code identification by Procter & Gamble.

## III. RATIONALE

MutaMouse is a transgenic mouse which has bacterial lacZ genes stably integrated into the genome of every cell. This gene is unexpressed in vivo but serves as a potential target for mutagenesis by chemicals, radiation or endogenous processes. The particular advantage of the transgene is that the lacZ gene is inserted within a lambda gt10 vector which can be rescued enzymatically from the mouse DNA. DNA isolated from any mouse tissue of choice is reacted with a lambda packaging extract in order to produce viable phage. Each lambda phage contains one lacZ target gene obtained from the mouse DNA. The phage are adsorbed to a large excess of E. coli C bacteria and allowed to multiply and form plaques on an agar surface. By including a chromogenic substrate in the agar for the lacZ gene produce, β-galactosidase, a dark blue color is developed in the wildtype (nonmutant) plaques. However, mutations that cause a loss or reduction in β-gal activity result in colorless or light blue plaques. The ratio of altered plaques to blue plaques is the lacZ mutant frequency in the mouse DNA being analyzed. This assay is sensitive to mutagenic agents that cause base-pair changes or deletions in regions of the lacZ target required for enzymatic activity. The construction of MutaMouse and its characteristics were described as strain 40.6 by Gossen et al., 1989.

## IV. MATERIALS

### A. Animal Tissue

Skin tissue samples for analysis were obtained by Procter & Gamble from MutaMouse transgenic mice, designated $CD_2$-lacZ80/HazfBR. Frozen tissue samples packaged in dry ice were sent to HWA, and the tissues were stored at -70°C or colder until removed for the extraction of DNA.

### B. Bacterial Host

The bacterial host for the development of plaques was E. coli C ($lac^-$, $tet^r$, $amp^r$) obtained from Dr. Jan Gossen, Medscand Ingeny, The Netherlands. Stocks were stored at -20°C or colder in 50% glycerol in Luria Bertani (LB) medium. Overnight cultures were subjected periodically to growth in LB medium containing ampicillin or tetracycline to guard against adventitious bacterial contamination. Daily cultures were prepared from overnight cultures in LB medium containing 0.2% maltose (LB/M medium). The daily cultures were grown in LB/M medium to a density of $OD_{600}$ = 0.5 to 1.0 prior to collection for use in the assay.

## EXPERIMENTAL DESIGN

### A. Sample Selection and Size

In this study, skin samples were the only tissue samples provided. The sponsor initially requested that 300,000 blue plaques ($lacZ^+$ gene population) be analyzed per sample. A number of samples were then selected for further analysis up to 600,000 $lacZ^+$ genes. Six coded skin samples were placed on hold and were not analyzed for this report (see Section I.B.)

### B. Mutation Assay

#### 1. Tissue preparation

Each sample of frozen skin was removed from -70°C storage and cut into small pieces with scissors. The pieces were then dispersed into lysis buffer (10 mM Tris•HCl, pH 8.0, 150 mM NaCl, 20 mM EDTA). Each dispersed tissue was digested for 2-3 hours at approximately 50°C in a shaking waterbath using 0.9% sodium dodecylsulfate and 0.9 mg/ml proteinase K in lysis buffer.

#### 2. DNA preparation

The tissue digest was mixed with an equal volume of equilibrated phenol:chloroform (1:1), using gentle inversion to produce a homogeneous suspension. The water phase (containing the DNA) was separated from the organic phase by centrifugation at approximately 1500 x g for 15 minutes. The water phase was then transferred to a clean tube, while being careful to avoid insoluble material at the interface.

A one-fifth volume of 8M potassium acetate was added to the water phase and mixed gently. Then one volume of chloroform was added and mixed by slow inversions. After centrifugation at approximately 2500 x g for 30 minutes at approx. 4°C, the upper aqueous phase was transferred to a clean tube. DNA was precipitated by the addition of 2 to 2.5 volumes of cold absolute ethanol.

The collected DNA was dissolved in TE-4 buffer (10 mM Tris•HCl, pH 7.5, 4 mM EDTA) and refrigerated indefinitely at approx. 4°C. The DNA concentration was determined from the ultraviolet absorbance at 260 nm, using the relationship of 70 µg/ml of calf thymus DNA for an absorbance of 1.0 (Rodriguez and Tait, 1983).

The molecular weight distribution of each DNA preparation was determined by electrophoresis in 0.5% agarose. The migration of the DNA and DNA molecular weight markers were visualized by ethidium bromide staining and photography of induced fluorescence. DNA preparations of good quality for the packaging reaction, defined as DNA remaining primarily between the origin and the 25,000 base-pair position, were used for the analysis of mutant frequency.

3. DNA packaging

Sequences of λgt10lacZ were removed from the mouse DNA and packaged into empty lambda preheads by use of lambda packaging extracts. The extracts were purchased commercially and consisted of two components (freeze/thaw extract and sonicate). The components were stored at -70°C or colder and were quickly thawed just prior to use.

Each DNA preparation was diluted with TE-4 buffer to a concentration of 1.5 μg DNA/μl. A volume of 5 μl was carefully pipeted into 10 μl of freeze/thaw extract and stirred. The sonicate (15 μl) was then immediately added and stirred. The reaction was incubated for approximately 3 hours at 37 ± 2°C, then terminated by the addition of 0.5 to 1 ml SM buffer (50 mM Tris·HCl, pH 7.5, 10 mM $MgSO_4$, 100 mM NaCl, 0.01% gelatin) and 30 μl of chloroform as preservative. The reaction mixture was pulse-spun to pellet the chloroform, and the preparation was stored in the dark under refrigeration (~4°C) until analyzed.

4. Lambda plaque production

The number of reliable lambda phage in each packaged DNA preparation was estimated by a titering. A measured volume was removed and incubated for 20-30 minutes at room temperature with 1 ml of E. coli C prepared as described below. LB/MM agarose medium was prepared, and 14 ml were added to the tube. After mixing, pouring the contents into a 150 mm plate, and allowing solidification, the culture was incubated overnight at approximately 37°C to allow plaques to develop. The plaques were counted and used to calculate the volume of packaged DNA preparation needed to produce approximately 1500 plaques.

The packaged DNA preparations from each tissue sample were analyzed by producing 100,000 or more plaques at a nominal (precalculated) density of approximately 1500 plaques per 150 mm culture plate. Usually, several packaging reactions and platings (on different days) were used to achieve the total population desired for each tissue sample. The plating procedure is described in the last paragraph of this section.

LB/MM agarose medium was prepared on the day of use. The contents were 0.75% purified agarose in LB medium to which 10 mM $MgSO_4$, 0.2% maltose, and 0.35 mg/ml X-gal were added aseptically to molten medium (approx. 55°C) just prior to use. The X-gal solution was previously dissolved in dimethylformamide (DMF) and then diluted into the agarose solution such that the DMF concentration was less than 0.5%.

E. coli C bacteria were obtained from daily cultures as described in Section IV.E. The cultures were centrifuged and resuspended in fresh LB/MM medium at $OD_{600}$ of approximately 0.40-0.60, which corresponds to approximately 2 to 4 x $10^8$ bacteria/ml. This suspension was held on ice and used within 1-2 hours of preparation.

A series of tubes was prepared for the adsorption of phage to the bacteria. Normally, three plates were poured from one adsorption, so the volume of packaged DNA preparation used corresponded to a titer of approximately 4500 phage. A 3 ml aliquot of bacterial suspension was added, and the suspension was mixed and incubated for 20-30 minutes at room temperature. LB/MM agarose (42 ml) was added and mixed. The contents were then quickly pipeted into three empty 150 mm culture plates at 15 ml/plate, spread evenly, and allowed to harden. The cultures were incubated overnight at approximately 37°C to allow plaques to develop.

E. Plate scoring

All cultures were scored on the day of removal from the incubator. The total population was determined from blue plaque counts on four representative plates. An area equivalent to one-tenth the plate area was marked on the plate surface and used as the count area. The average plaque count and standard deviation were determined. The total population was calculated as the average blue plaque count x 10 x the number of plates scored for mutant plaques.

Each plate was examined carefully for presumptive mutant plaques, identified as colorless plaques or plaques with a reduced color relative to the majority of blue, wildtype plaques. The positions were marked and the plate given to a second person to locate any additional presumptive mutants.

All presumptive mutant plaques were picked by collecting a core through the plaque with a clean Pasteur pipet. The contents were transferred to a microfuge tube containing 300 µl SM buffer and 50 µl chloroform. After mixing well, the phage preparations were allowed to sit for several hours or overnight in a refrigerator. The phage preparations can be held

indefinitely at approximately 4°C in the dark but were assayed for mutant content within 7 days to avoid possible loss of infectivity.

6. Mutant confirmation

Presumptive mutants were tested for mutant phenotypes by replating onto E. coli C cultures containing λ-gal. Bacterial suspensions in LB/MM agarose were prepared as already described in Section V.B.4, except that phage were not included. Either 150 mm plates or the much larger 24 cm x 24 cm bottom agar plates were used. In the latter case, 30 ml of LB/MM agarose were mixed with 3 ml of bacteria suspension and poured for each plate. After hardening, the plates were positioned on a grid pattern of approximately 15 mm x 15 mm. A 10 μl aliquot of each presumptive mutant phage preparation was applied to one grid area. After the spots had adsorbed into the agarose, the plates were incubated overnight at approximately 37°C.

A presumptive mutant phage sample was scored as mutant if any colorless or light blue plaques were found in the spotted area. An all-blue response of wildtype intensity in the spotted area was scored as negative for mutation.

If the spotted area was completely lysed so that individual plaques were not distinguishable, a second confirmation test with a smaller titer was performed. Conversely, if no plaques were observed, a second trial with a larger titer was used for that phage preparation.

7. Mutant frequency calculation

The mutant frequency (MF) in a DNA sample was defined as the ratio of confirmed mutant plaques to the total plaques analyzed. The MF was expressed in units of $10^6$ plaques. To increase the population of analyzed lacZ genes for a given skin DNA sample, the results from several packaging reactions and phage platings were combined and used for the MF calculation.

C. Data Presentation

For each skin DNA sample analyzed, the following data are reported: the total number of plaques scored, the number of colorless mutant plaques found, the number of color-mutant plaques, the total number of mutant plaques, and the calculated mutant frequency.

D. Assay Acceptance Criteria

The assay was considered acceptable for evaluation of the results by meeting the following criteria:

- The negative control tissue yielded a mutant frequency consistent with historical experience for that tissue. The average mutant frequency among the 6 negative control samples was (40 ± 11) x $10^{-6}$, which was not significantly different from the HWA historical negative control data provided below:

HWA HISTORICAL NEGATIVE CONTROL
MUTANT FREQUENCY IN SKIN

| ANIMAL* | MUTANT LACZ GENES | WILDTYPE LACZ GENES | MUTANT FREQ. x $10^6$ |
|---|---|---|---|
| 1 | 29 | 1,059,759 | 27.4 |
| 2 | 28 | 1,013,281 | 27.6 |
| 3 | 37 | 1,062,595 | 34.8 |
| 4 | 32 | 1,092,815 | 29.3 |
| 5 | 24 | 1,156,650 | 20.7 |
| 6 | 28 | 1,204,389 | 23.2 |
| 7 | 34 | 1,050,676 | 32.4 |

* Male, 8-10 weeks  Avg. MF = (27.9 ± 4.9) x $10^{-6}$

- The plaque density on the cultures scored for mutants did not exceed 2000 plaques (to 5% precision) per 150 mm plate.

- The efficiency of the packaging reaction for each DNA sample did not fall below 10,000 pfu/$\mu$g DNA. Because most DNA samples were packaged in several individual reactions, an occasional reaction in the 9000 pfu/$\mu$g DNA range was acceptable if the majority of plaques were obtained at higher efficiency.

E. Response Evaluation Criteria

All tissue samples were analyzed at plaque populations greater than 100,000 and in accordance with directives from the sponsor. Because the concurrent negative controls were comparable to the HWA historical negative control data, the concurrent negative controls were used to evaluate the responses in the treated tissues.

An appropriate statistical method for data analysis is not yet developed. Therefore, a guideline of at least a 2-fold increase in mutant frequency over the concurrent negative control mean was used as a minimum response to indicate mutagenic activity. In this study, that mutant frequency was 80 x $10^{-6}$ or greater. In addition, a consistently elevated response among the replicate samples was necessary to consider a treatment to be mutagenic.

## VI. RESULTS AND DISCUSSION

All of the skin samples evaluated in this study were coded. Upon completion of the study, the code was broken by the sponsor, and the data were arranged into the appropriate treatment groups for analysis, as shown in Table 1.

The results in Table 1 show that the negative control skin samples, harvested 7 days after initiation of the study, yielded mutant frequencies within the HWA historical distribution of $(28 \pm 5) \times 10^{-6}$. Higher mutant frequency values were obtained at 10 days after study initiation. It is not clear why this second set of controls should yield apparently higher mutant frequencies, but if all six control animals are considered together, the resultant mean and standard deviation, $(40 \pm 11) \times 10^{-6}$, was not significantly different from the HWA historical distribution obtained from approximately 1,000,000 plaques per animal. Hence, the concurrent negative control, consisting of all 6 samples, was considered to be appropriate for the evaluations of the treated skin samples.

7,12-Dimethylbenz(a)anthracene (DMBA) was used as a positive control test substance. As shown in Table 1, the DMBA treatment caused only a minimal increase in mutant frequency by 4 days after application. However, by 7 days after treatment, a large increase in mutant frequency was observed. This response increased somewhat further to an average mutant frequency of $539 \times 10^{-6}$ at 10 days. (One of the animals, coded 4D1, showed a mutant frequency that was indicative of a mutant offspring in the animal breeding program, so this animal was excluded from the analysis of DMBA-induced mutagenesis.) These results demonstrated the mutagenicity of a well-known chemical mutagen and showed the importance of harvest time in detecting this activity. The time course for the maximum accumulation of induced mutants in skin is not established for any chemical, but 7-10 days' harvest time appears to approach the time necessary for maximum assay sensitivity.

Compound G0539.06 did not induce any significant increases in mutant frequency when assayed 7 and 10 days after treatment. The mutant frequencies in the skin samples from individual animals ranged from $16.6 \times 10^{-6}$ to $42.2 \times 10^{-6}$, which fell within the negative control range of frequencies. The lack of any response at 7 or 10 days after treatment indicated that G0539.06 did not possess any significant mutagenic activity in mouse skin.

Compound R0363.01 was analyzed 7 days after treatment. Again, no departure from the range of mutant frequencies typical of negative control skins were observed. Thus, no evidence for mutagenic activity was obtained for this test substance.

## VII. CONCLUSION

MutaMouse skin samples assayed 7 and 10 days after the application of 7,12-dimethylbenz(a)anthracene showed large increases in the lacZ mutant frequency. In contrast, test compound G0539.06 did not induce any increases in mutant frequency at these harvest times. Similarly, test compound R0363.01 caused no change in mutant frequency at 7 days following treatment. Thus, no evidence for mutagenic activity in skin was obtained for either test compound in this study.

## VIII. REFERENCES

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Rodriguez, R.L. and Tait, R.C.: Recombinant DNA Techniques: An Introduction, The Benjamin/Cummings Publishing Company, Inc., Menlo Park, CA, 1983, pp 42-43.

# IX. EXPERIMENTAL DATA TABLES

TABLE 1

LacZ MUTANT FREQUENCY IN MUTAMOUSE SKIN SAMPLES

P&G Study No.: B91-0227
P&G Reference No.: 91000672
HWA Assay No.: 14654

| Tissue Sample Identity[a] | Packaging Reaction No.[b] | Packaging Reaction Titer (pfu/μg DNA) | Plating Density (LacZ⁺ plaques/plate) | Total LacZ⁺ Population | Colorless LacZ Mutants | Colored LacZ Mutants | Total LacZ Mutants | MF x 10⁻⁶ |
|---|---|---|---|---|---|---|---|---|
| 1B1 Negative Control, 7 days | 1633-1 | 36,027 | 1163 | 87,225 | 1 | 0 | 1 | |
| | 1633-1 | 36,027 | 1543 | 46,290 | 1 | 0 | 1 | |
| | 1633-1 | 36,027 | 1215 | 21,870 | 2 | 1 | 3 | |
| | 1633-2 | 18,827 | 1580 | 142,200 | 1 | 1 | 2 | |
| | 1633-3 | 20,507 | 1680 | 151,200 | 3 | 2 | 5 | |
| | 1633-4 | 22,320 | 1365 | 139,230 | 2 | 0 | 2 | |
| | | | | 588,015 | | | 14 | 23.8 |
| 1B2 Negative Control, 7 days | 1633-1 | 21,413 | 1363 | 122,670 | 3 | 0 | 3 | |
| | 1633-2 | 12,960 | 1515 | 99,990 | 1 | 0 | 1 | |
| | 1633-3 | 14,747 | 1535 | 96,705 | 4 | 0 | 4 | |
| | 1633-4 | 16,880 | 1428 | 114,240 | 5 | 1 | 6 | |
| | 1633-5 | 17,787 | 1595 | 130,790 | 5 | 0 | 5 | |
| | 1633-6 | 21,493 | 1835 | 55,050 | 1 | 0 | 1 | |
| | | | | 619,445 | | | 20 | 32.3 |
| 1B3 Negative Control, 7 days | 1633-1 | 23,573 | 1323 | 119,070 | 2 | 1 | 3 | |
| | 1633-2 | 16,907 | 1420 | 113,600 | 3 | 1 | 4 | |
| | 1633-3 | 18,347 | 1385 | 70,635 | 1 | 0 | 1 | |
| | 1633-4 | 32,640 | 1448 | 221,544 | 10 | 2 | 12 | |
| | 1633-5 | 11,440 | 1240 | 66,960 | 2 | 0 | 2 | |
| | | | | 591,809 | | | 22 | 37.2 |

TABLE 1 (Continued)

LacZ MUTANT FREQUENCY IN MUTAMOUSE SKIN SAMPLES

| Tissue Sample Identity[a] | Packaging Reaction No.[b] | Packaging Reaction Titer (pfu/μg DNA) | Plating Density (LacZ⁺ plaques/plate) | Total LacZ⁺ Population | Colorless LacZ Mutants | Colored LacZ Mutants | Total LacZ Mutants | MF x $10^6$ |
|---|---|---|---|---|---|---|---|---|
| IC1 Negative Control, 10 days | 1670-1 | 18,853 | 1383 | 121,704 | 9 | 0 | 9 | |
| | 1670-2 | 20,000 | 1293 | 116,370 | 5 | 5 | 10 | |
| | 1670-3 | 14,560 | 2005 | 138,345 | 3 | 0 | 3 | |
| | 1670-4 | 13,893 | 1588 | 104,808 | 7 | 0 | 7 | |
| | 1670-5 | 14,773 | 1575 | 66,150 | 1 | 1 | 2 | |
| | 1670-5 | 14,773 | 1443 | 43,290 | 1 | 1 | 2 | |
| | | | | 590,667 | | | 33 | 55.9 |
| IC2 Negative Control, 10 days | 1670-1 | 23,307 | 1435 | 159,285 | 5 | 5 | 10 | |
| | 1670-2 | 13,467 | 1505 | 96,320 | 4 | 1 | 5 | |
| | 1670-3 | 19,573 | 1578 | 63,120 | 1 | 0 | 1 | |
| | 1670-3 | 19,573 | 1383 | 53,937 | 1 | 1 | 2 | |
| | 1670-4 | 11,093 | 1370 | 69,870 | 1 | 0 | 1 | |
| | 1670-5 | 25,573 | 1448 | 169,416 | 9 | 0 | 9 | |
| | | | | 611,948 | | | 28 | 45.8 |
| IC3 Negative Control, 10 days | 1670-1 | 32,613 | 1188 | 163,944 | 1 | 4 | 5 | |
| | 1670-2 | 23,547 | 1093 | 65,580 | 2 | 1 | 3 | |
| | 1670-2 | 23,547 | 1118 | 60,372 | 1 | 2 | 3 | |
| | 1670-3 | 11,813 | 1555 | 88,635 | 3 | 0 | 3 | |
| | 1670-4 | 13,627 | 1438 | 99,222 | 4 | 4 | 8 | |
| | 1670-5 | 10,107 | 1790 | 85,920 | 3 | 1 | 4 | |
| | 1670-6 | 9,787 | 1450 | 47,850 | 0 | 2 | 2 | |
| | | | | 611,523 | | | 28 | 45.8 |

TABLE 1 (Continued)

LacZ MUTANT FREQUENCY IN MUTAMOUSE SKIN SAMPLES

| Tissue Sample Identity[a] | Packaging Reaction No.[b] | Packaging Reaction Titer (pfu/μg DNA) | Plating Density (LacZ⁺ plaques/plate) | Total LacZ⁺ Population | Colorless LacZ Mutants | Colored LacZ Mutants | Total LacZ Mutants | MF x $10^6$ |
|---|---|---|---|---|---|---|---|---|
| 4A1 | 1670-1 | 20,800 | 1165 | 118,830 | 6 | 0 | 6 | |
| DMBA, | 1670-2 | 10,667 | 1350 | 68,850 | 5 | 2 | 7 | |
| 4 days | 1670-3 | 16,293 | 1585 | 128,385 | 8 | 2 | 10 | |
| | | | | 316,065 | | | 23 | 72.8 |
| 4A2 | 1670-1 | 19,520 | 1375 | 132,000 | 12 | 1 | 13 | |
| DMBA, | 1670-2 | 12,907 | 1423 | 89,649 | 12 | 2 | 14 | |
| 4 days | 1670-3 | 9,707 | 1483 | 66,735 | 8 | 2 | 10 | |
| | | | | 288,384 | | | 37 | 128.3 |
| 4A3 | 1670-1 | 22,267 | 1228 | 136,308 | 5 | 0 | 5 | |
| DMBA, | 1670-2 | 12,640 | 1375 | 86,625 | 3 | 1 | 4 | |
| 4 days | 1670-3 | 9,867 | 1550 | 69,750 | 3 | 2 | 5 | |
| | | | | 292,683 | | | 14 | 47.8 |

TABLE 1 (Continued)

LacZ MUTANT FREQUENCY IN MUTAMOUSE SKIN SAMPLES

| Tissue Sample Identity[a] | Packaging Reaction No.[b] | Packaging Reaction Titer (pfu/μg DNA) | Plating Density (LacZ⁻ plaques/plate) | Total LacZ⁻ Population | Colorless LacZ Mutants | Colored LacZ Mutants | Total LacZ Mutants | MF x 10⁶ |
|---|---|---|---|---|---|---|---|---|
| 4B2 DMBA, 7 days | 1633-1 | 34,587 | 1295 | 194,250 | 65 | 4 | 69 | |
| | 1633-2 | 24,747 | 1410 | 118,440 | 59 | 9 | 68 | |
| | | | | 312,690 | | | 137 | 438.1 |
| 4D1 DMBA, 7 days | 1633-1 | 28,720 | 1035 | 139,725 | 973 | 4 | 977 | 6992 |
| 4D2 DMBA, 7 days | 1633-1 | 19,360 | 1513 | 139,196 | 72 | 15 | 87 | |
| | 1633-2 | 17,280 | 1533 | 128,772 | 58 | 8 | 66 | |
| | 1633-3 | 31,813 | 1498 | 44,940 | 22 | 4 | 26 | |
| | | | | 312,908 | | | 179 | 572.1 |

TABLE 1 (Continued)

LacZ MUTANT FREQUENCY IN MUTAMOUSE SKIN SAMPLES

| Tissue Sample Identity[a] | Packaging Reaction No.[b] | Packaging Reaction Titer (pfu/μg DNA) | Plating Density (LacZ⁺ plaques/plate) | Total LacZ⁺ Population | Colorless LacZ Mutants | Colored LacZ Mutants | Total LacZ Mutants | MF x $10^6$ |
|---|---|---|---|---|---|---|---|---|
| 4C1 DMBA, 10 days | 1670-1 | 33,307 | 1203 | 191,277 | 83 | 6 | 89 | |
| | 1670-2 | 27,440 | 1375 | 94,875 | 45 | 14 | 59 | |
| | | | | 286,152 | | | 148 | 517.2 |
| 4C2 DMBA, 10 days | 1670-1 | 24,880 | 1268 | 148,356 | 82 | 23 | 105 | |
| | 1670-2 | 23,360 | 1370 | 152,070 | 75 | 13 | 88 | |
| | | | | 300,426 | | | 193 | 642.4 |
| 4C3 DMBA, 10 days | 1670-1 | 22,453 | 1195 | 118,305 | 60 | 11 | 71 | |
| | 1670-2 | 13,200 | 1435 | 94,710 | 27 | 9 | 36 | |
| | 1670-3 | 18,400 | 1210 | 22,990 | 6 | 2 | 8 | |
| | 1670-3 | 18,400 | 1355 | 36,585 | 14 | 4 | 18 | |
| | 1670-4 | 17,493 | 1455 | 34,920 | 6 | 2 | 8 | |
| | | | | 307,510 | | | 141 | 458.5 |

TABLE 1 (Continued)

LacZ MUTANT FREQUENCY IN MUTAMOUSE SKIN SAMPLES

| Tissue Sample Identity[a] | Packaging Reaction No.[b] | Packaging Reaction Titer (pfu/μg DNA) | Plating Density (LacZ⁺ plaques/plate) | Total LacZ⁺ Population | Colorless LacZ Mutants | Colored LacZ Mutants | Total LacZ Mutants | MF x 10⁻⁶ |
|---|---|---|---|---|---|---|---|---|
| 2B1 Compound G0539.06, 7 days | 1633-1 | 18,160 | 1190 | 107,100 | 2 | 0 | 2 | |
| | 1633-2 | 23,787 | 1353 | 154,242 | 2 | 0 | 2 | |
| | 1633-3 | 14,053 | 1578 | 47,340 | 2 | 0 | 2 | |
| | 1633-3 | 14,053 | 1598 | 43,146 | 0 | 1 | 1 | |
| | 1633-4 | 12,480 | 953 | 57,180 | 0 | 0 | 0 | |
| | 1633-5 | 13,147 | 1795 | 114,880 | 1 | 0 | 1 | |
| | 1633-6 | 15,920 | 1515 | 36,360 | 0 | 0 | 0 | |
| | 1633-7 | 9,093 | 1425 | 42,750 | 2 | 0 | 2 | |
| | | | | 602,998 | | | 10 | 16.6 |
| 2B2 Compound G0539.06, 7 days | 1633-1 | 36,560 | 1100 | 99,000 | 1 | 0 | 1 | |
| | 1633-1 | 36,560 | 1283 | 107,772 | 6 | 1 | 7 | |
| | 1633-2 | 18,053 | 1435 | 93,275 | 6 | 0 | 6 | |
| | 1633-3 | 26,747 | 1700 | 212,500 | 6 | 1 | 7 | |
| | 1633-4 | 23,173 | 1625 | 156,000 | 5 | 0 | 5 | |
| | | | | 668,547 | | | 26 | 38.9 |
| 2B3 Compound G0539.06, 7 days | 1633-1 | 21,040 | 1253 | 72,674 | 3 | 1 | 4 | |
| | 1633-2 | 17,813 | 1515 | 136,350 | 4 | 0 | 4 | |
| | 1633-3 | 51,600 | 1430 | 102,960 | 4 | 2 | 6 | |
| | 1633-3 | 51,600 | 1733 | 181,965 | 3 | 1 | 4 | |
| | 1633-4 | 21,440 | 1790 | 179,000 | 3 | 2 | 5 | |
| | | | | 672,949 | | | 23 | 34.2 |

TABLE 1 (Continued)

LacZ MUTANT FREQUENCY IN MUTAMOUSE SKIN SAMPLES

| Tissue Sample Identity[a] | Packaging Reaction No.[b] | Packaging Reaction Titer (pfu/μg DNA) | Plating Density (LacZ⁺ plaques/plate) | Total LacZ⁺ Population | Colorless LacZ Mutants | Colored LacZ Mutants | Total LacZ Mutants | MF x 10⁻⁶ |
|---|---|---|---|---|---|---|---|---|
| 2C1 Compound G0539.06, 10 days | 1670-1 | 20,960 | 1178 | 123,690 | 2 | 0 | 2 | |
| | 1670-2 | 19,173 | 1333 | 123,969 | 3 | 1 | 4 | |
| | 1670-3 | 10,000 | 1433 | 61,619 | 1 | 1 | 2 | |
| | 1670-4 | 10,000 | 1695 | 81,360 | 0 | 1 | 1 | |
| | 1670-5 | 11,093 | 1950 | 101,400 | 2 | 1 | 3 | |
| | 1670-6 | 13,280 | 1723 | 108,549 | 2 | 3 | 5 | |
| | | | | 600,587 | | | 17 | 28.3 |
| 2C2 Compound G0539.06, 10 days | 1670-1 | 19,413 | 863 | 80,259 | 1 | 1 | 2 | |
| | 1670-2 | 21,920 | 1463 | 149,226 | 2 | 0 | 2 | |
| | 1670-3 | 13,147 | 1845 | 105,165 | 2 | 0 | 2 | |
| | 1670-4 | 21,067 | 1918 | 195,636 | 7 | 4 | 11 | |
| | 1670-5 | 15,333 | 1458 | 109,350 | 0 | 1 | 1 | |
| | 1670-6 | 13,253 | 1623 | 68,166 | 3 | 0 | 3 | |
| | | | | 707,802 | | | 21 | 29.7 |
| 2C3 Compound G0539.06, 10 days | 1670-1 | 22,133 | 1335 | 128,160 | 4 | 0 | 4 | |
| | 1670-2 | 10,000 | 1763 | 84,624 | 3 | 0 | 3 | |
| | 1670-3 | 17,520 | 968 | 78,408 | 5 | 0 | 5 | |
| | 1670-4 | 22,347 | 1533 | 133,371 | 5 | 2 | 7 | |
| | 1670-5 | 26,453 | 1540 | 190,960 | 5 | 2 | 7 | |
| | | | | 615,523 | | | 26 | 42.2 |

TABLE 1 (Continued)

LacZ MUTANT FREQUENCY IN MUTAMOUSE SKIN SAMPLES

| Tissue Sample Identity[a] | Packaging Reaction No.[b] | Titer (pfu/μg DNA) | Plating Density (LacZ⁻ plaques/plate) | Total LacZ⁻ Population | Colorless LacZ Mutants | Colored LacZ Mutants | Total LacZ Mutants | MF x $10^6$ |
|---|---|---|---|---|---|---|---|---|
| 3D1 | 1633-1 | 31,653 | 1375 | 206,250 | 2 | 0 | 2 | |
| Compound | 1633-2 | 13,813 | 1320 | 89,760 | 2 | 3 | 5 | |
| R0363.01, | 1633-3 | 12,933 | 1663 | 89,802 | 1 | 1 | 2 | |
| 7 days | 1633-4 | 10,693 | 1848 | 116,424 | 1 | 0 | 1 | |
| | 1633-5 | 17,893 | 1425 | 125,400 | 4 | 0 | 4 | |
| | | | | 627,636 | | | 14 | 22.3 |
| 3D2 | 1633-1 | 31,067 | 1225 | 180,075 | 6 | 0 | 6 | |
| Compound | 1633-2 | 14,933 | 1568 | 114,464 | 3 | 2 | 5 | |
| R0363.01, | 1633-3 | 24,480 | 1870 | 220,660 | 2 | 0 | 2 | |
| 7 days | 1633-4 | 14,000 | 1743 | 113,295 | 2 | 0 | 2 | |
| | | | | 628,494 | | | 15 | 23.9 |
| 3D3 | 1633-1 | 15,733 | 1290 | 96,750 | 7 | 0 | 7 | |
| Compound | 1633-2 | 12,907 | 1438 | 89,156 | 1 | 1 | 2 | |
| R0363.01, | 1633-3 | 17,627 | 1653 | 138,852 | 5 | 0 | 5 | |
| 7 days | 1633-4 | 14,000 | 1888 | 130,272 | 1 | 0 | 1 | |
| | 1633-5 | 16,000 | 1533 | 119,574 | 9 | 0 | 9 | |
| | | | | 574,604 | | | 24 | 41.8 |

[a] The test sample code, experimental identity revealed after analysis, and the harvest times (days after dosing) are given.

[b] Packaging reaction number: Gigapack II Gold lot number (nnnn) and the individual reactions performed (-n) for each tissue sample.

pfu = plaque forming unit  MF = LacZ Mutant Frequency

DMBA = 7,12-Dimethylbenz(a)anthracene, 100 μg per animal, used as positive control.